Veterinary Integrative Sciences 2026; 24(1): e2026004-1-12. DOI: 10.12982/VIS.2026.004

Veterinary Integrative Sciences
ISSN; 2629-9968 (online)

Research article

Draft genome sequences of multidrug resistant *Staphylococcus arlettae* isolated from cow nasal swab at Kelantan Malaysia Farm

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Abstract

Staphylococcus arlettae is an emerging opportunistic pathogen associated with bovine mastitis, a significant concern in animal health and milk production. This study investigates the genomic characteristics of a multidrug-resistant *S. arlettae* strain isolated from a healthy cow in Malaysia, providing crucial insights into its potential for pathogenicity and spread. The *Staphylococcus arlettae* BK2L15 isolate was obtained from a nasal swab of a healthy cow in Kelantan. Initial identification was based on its growth characteristics on Mannitol Salt Agar, followed by antimicrobial susceptibility testing using the disc diffusion method. Subsequent identification with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) confirmed the isolate as *Staphylococcus arlettae*, with a score value of 2.09. Whole genome sequencing was conducted using the Illumina MiSeq system, with raw read quality evaluated through QUAST and genome assembly performed using SPAdes v3.12.0. The *Staphylococcus arlettae* BK2L15 exhibited a multidrug resistance phenotype towards Erythromycin, Cefoxitin, Fusidic Acid, Oxacillin, Penicillin, Azithromycin and Amoxicillin. Whole genome analysis revealed a genome size of 2,699,512 bp with a GC content of 33.50%, assembled into two contigs. The genome comprises 2,915 protein-coding sequences includes all genomes features. The sequencing data of this strain provide a valuable reference for future fine-scale comparative genomic studies, facilitating the establishment of genomic relationships between lineages and enabling the prediction of virulence factors, mobile genetic elements, and antimicrobial resistance genes.

Keywords: Multidrug resistant, *Staphylococcus arlettae*, Whole Genome Sequence.

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Article history; *received manuscript: 4 September 2024, revised manuscript: 25 October 2024, accepted manuscript: 31 January 2025, published online: 11 February 2025,*
Academic editor; *Prapas Patchanee*

INTRODUCTION

Staphylococcus arlettae (SAR), a coagulase-negative staphylococcus (CoNS), has been isolated from diverse environments, including livestock, humans, and clinical settings (Bhardwaj et al., 2016; Han et al., 2023; Kherdekar., 2023). In dairy farms, SAR has been identified as an emerging pathogen contributing to bovine mastitis and intramammary infections in dairy goats. The presence of SAR in farm animals poses a significant risk to livestock health and farm productivity. Despite its growing importance in veterinary medicine, the distribution and role of SAR in dairy farms across Malaysia remain largely unexplored. Understanding the prevalence and pathogenic mechanisms of SAR in this region is crucial for effective disease management.

The increasing antimicrobial resistance (AMR) of SAR has become a critical concern, particularly in environments with high antibiotic usage. SAR can acquire and disseminating antibiotic resistance genes, contributing to the emergence of multidrug-resistant (MDR) strains (Xu et al., 2015; Andreis et al., 2017; Liu et al., 2017; Nobrega et al., 2018). For instance, SAR strains isolated from poultry and livestock have been found to harbor plasmids encoding multiple resistance genes, including those conferring resistance to macrolides, tetracyclines, and beta-lactams. The detection of the *mec*A gene, associated with methicillin resistance, further highlights the clinical and veterinary implications of SAR (Nascimento et al., 2005; Liu et al., 2017). In Malaysia, the lack of comprehensive data on the antimicrobial susceptibility profiles of SAR isolates underscores the urgency for targeted surveillance and antimicrobial stewardship practices.

Whole genome sequencing (WGS) has emerged as an essential tool for studying the genetic architecture, resistance mechanisms, and virulence factors of MDR pathogens like SAR. Recent research has utilized WGS to uncover the evolutionary dynamics and genomic diversity of SAR strains isolated from various hosts, including livestock and humans. Globally, only 59 genome sequences of SAR have been deposited in public databases, with just five complete genomes submitted between 2019 and 2022 (Yu et al., 2019; Wong et al., 2022). These genomes have provided insights into SAR's potential as a reservoir of resistance genes and its ecological adaptability. Despite its importance, no WGS data for SAR isolates from Malaysia are currently available, leaving a significant gap in our understanding of the pathogen in this region.

The scarcity of genomic and molecular data on *S. arlettae* (SAR) isolates in Malaysia presents a significant obstacle to understanding the epidemiology and pathogenic potential of this emerging opportunistic pathogen. While limited genomic studies have indicated a role for SAR in antimicrobial resistance and virulence, a comprehensive analysis of Malaysian isolates is lacking. This study hypothesizes that SAR isolates from Malaysian dairy farms possess unique genetic determinants that contribute to their adaptation and persistence within the local environment. To address this knowledge gap, this study will employ a comprehensive approach to characterize *S. arlettae* isolates from Malaysian dairy farms, encompassing phenotypic and genotypic analyses. This will involve conducting antimicrobial susceptibility testing to determine resistance profiles, followed by molecular characterization to identify the presence of specific resistance and virulence genes. Finally, whole-genome sequencing will be utilized to analyze the genomic architecture and evolutionary relationships of these isolates, providing a deeper understanding of their adaptive mechanisms and pathogenic potential. The findings generated from these objectives will provide crucial data for enhancing surveillance, detection, and control strategies for SAR in dairy farms, ultimately contributing to improved animal health and production outcomes.

The limited availability of SAR genomic data, coupled with its emerging role in antimicrobial resistance and livestock infections, necessitates further investigation. By bridging this knowledge gap, this study aims to contribute

valuable insights into the genomic and molecular characteristics of SAR, enhancing our understanding of its impact on livestock health and public health in Malaysia.

MATERIALS AND METHODS

Bacterial isolation and identification

Staphylococcus arlettae BK2L15 was isolated from nasal swab of a healthy cow at Kampung Panjang Perupok, Bachok, Kelantan. This isolate was grown on Mannitol Salt Agar overnight at 37C for 24 hours. The colony was identified as small and off- white colonies and the medium color changed from phenol red to yellow as it can ferment the mannitol present in the agar medium. Isolates was genotypically identified by using PCR identification of *nuc* and *mec*A genes as described by Suhaili et al. (2018).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) Phenotypic identification

A pure culture isolate of a white colony was selected for spectra analysis using the MALDI-TOF Biotyper 3.0 database Ultraflex platform (Bruker Daltonics, Bremen, Germany). Sample preparation for each isolate involved suspending a single colony of bacteria in 500 ml of 70% ethanol, which was then centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet was air-dried before being resuspended by adding 25 ml of 70% formic acid solution. The bacterial suspension was gently mixed, and then 25 ml of pure acetonitrile was added to the mixture. The suspension was centrifuged for 2 minutes at 13,000 rpm to extract protein from the pellet. One milliliter of crude protein extract was transferred onto a 96-spot polished steel target plate (Bruker Daltonics) and allowed to dry at room temperature. The samples were then covered with 1 ml of α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics). Positive control and calibration reference were performed using 1 ml of bacterial test standard (BTS; Bruker Daltonics). The mass range analysis of the spectra was conducted using the MicroFlex tool (Bruker Daltonics) in positive linear mode, covering a range of 2000-20,000 m/z. Each spectrum was automatically acquired after 300 laser shots, in accordance with the system's procedure. Microorganism identification was performed in duplicate by analyzing the score, which reflects the degree of similarity with the reference spectrum in the database. A MALDI Biotyper score of 2.000-2.299 suggests secure genus identification or probable species identification, while a score of 2.300-3.000 indicates highly probable species identification. The main spectrum was obtained using MALDI Biotyper automated FlexControl software version 3.0 (Bruker Daltonics).

Antibiotic susceptibility testing and Multiple Antibiotic Resistance (MAR) Index

The antimicrobial susceptibility testing of the isolates was conducted using the Kirby– Bauer disc diffusion method, which is in accordance with the guidelines set by the Clinical and Laboratory Standard Institute (CLSI) and British Society for Antimicrobial Chemotherapy (BSAC) (BSAC, 2001; CLSI, 2023). For determining methicillin resistance, 1 µg of oxacillin and 30 µg of cefoxitin discs (Oxoid, UK) were utilized alongside reference strains S. aureus ATCC 25923 as positive controls (Magiorakos et al., 2012). The results of the susceptibility testing towards other antibiotics are documented in Table 1, following the recommendations of Magiorakos et al. (2012) with some adjustment. The diameter of the inhibition zone was measured in accordance with the CLSI and BSAC guidelines (BSAC 2001; CLSI, 2023).

The MAR index was calculated for each isolate using the formula MAR = a/b, where 'a' represents the number of antibiotics to which the test isolate exhibited resistance, and 'b' denotes the total number of antibiotics tested for susceptibility in the isolate.

Genome submissions to NCBI GenBank

The genome sequence of the 2,699,512 bp *Staphylococcus arlettae* BK2L15 characterized in this study has been deposited at GenBank under the accession number JBDLNW000000000.

Genome Sequencing, Assembly and Annotation

The genome sequencing of *Staphylococcus arlettae* BK2L15 was carried out using the Illumina Miseq platform, which generated 150-bp paired-end reads. The paired-end reads were then trimmed using the BV-BRC Fastq Utilities combines pipelines, which involved the removal of adapters and a quality check using Fastqc (https://www.bv- brc.org/app/FastqUtil). The trimmed reads were then assembled using Unicycler version v0.4.8, which utilized the SPAdes optimizer (v3.14.2) with 127 k-mer sizes to produce output in the form of fasta files containing contigs (Wick et al., 2017). The assembled file was subsequently validated and annotated using the Rapid Annotation Subsystems Technology (RAST) (Brettin et al., 2015).

Comprehensive Genome Analysis

The genome analysis of the assembly file was conducted through the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org). The analysis included a comprehensive comparative study that provided a subsystem summary, genomic features, and a phylogenetic tree, all of which distinguished the genome from its closest neighbors. The results of the analysis included a genome quality assessment, identification of AMR and virulence genes, specialty genes, a subsystem overview, and a list of features that distinguished the genome from its nearest neighbor. The identification of AMR genes is analyzed using Bacterial and Viral Bioinformatic Resource Centre (BV-BRC). The study also provided a list of the closest genome sequences and a phylogenetic tree that demonstrated the evolutionary relationships between the genomes (Davis et al., 2020). Species identification was carried prior before comprehensive genome analysis by using polymerase chain reaction (PCR).

Ethical Approval

Informed written and signed consent was obtained from animal owners or their representatives. This research was reviewed and approved on 27th October 2022 by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (Reference: UMK/FPV/ACUE/PG/003/2022)

Funding information

This work was funded by a Fundamental Research Grant Scheme (FRGS), Ministry of Higher Education of Malaysia under FRGS/1/2020/WAB04/UNISZA/03/2.

RESULTS

Bacterial identification and antimicrobial susceptibility test

This isolate was identified as *Staphylococcus arlettae* based on its phenotypic characteristics, including the appearance of small, off-white colonies on mannitol salt agar, with no color change in the medium, indicating the absence of mannitol fermentation. Genotypic identification via PCR confirmed that the

isolate was negative for the nuc gene but positive for the *mecA* gene. Further identification using MALDI- TOF mass spectrometry, with a score of 2.09 provided by the Biotyper 3.0 database on the Ultraflex platform, confirmed the isolate as *Staphylococcus arlettae*. The MALDI- TOF score of 2.09 suggests a high-confidence identification of the *Staphylococcus arlettae* BK2L15 genome.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test results indicate that the isolate is resistant to erythromycin, cefoxitin, fusidic acid, oxacillin, penicillin, azithromycin and amoxicillin, but is susceptible to ciprofloxacin, tigecycline, tetracycline, and other antibiotics listed in Table 1. This pattern helps clinicians in selecting the most effective antibiotic for treatment. The Multiple Antibiotic Resistance (MAR) index, calculated as the ratio of the number of antibiotics to which the isolate is resistant to the total number of antibiotics tested, was determined to be 0.4.

Table 1 Antimicrobial Susceptibility Test of *Staphylococcus arlettae* BK2L15

Antimicrobial used	Interpretation *(R | I | S)
Erythromycin	R
Ciprofloxacin	S
Clindamycin	I
Cefoxitin	R
Fusidic Acid	R
Gentamycin	S
Tetracycline	S
Tigercycline	S
Minocycline	S
Oxacillin	R
Teicoplanin	S
Penicillin	R
Linezolid	S
Rifampicin	S
Azithromycin	R
Amoxicillin	R

*R: Resistant; I: Intermediate; S: Susceptible

Features of the Staphylococcus arlettae BK2L15 genome

This assembled genome had 2 contigs, with the total length of 2,699,512 bp and an average G+C content of 33.50%. General genome features are shown in Table 2.

Table 2 General genome features of Staphylococcus arlettae BK2L15 generated using BV-BRC Bioinformatics tools online accessible.

Feature	Value
Contigs	2
GC content (%)	33.50
Genome Length	2,699,512
Total of protein-coding sequences (CDSs)	2,915
Number of ribosomal RNA genes	22
N50	2,668,290

Subsystem Analysis of *Staphylococcus arlettae* BK2L15

A subsystem is a collection of proteins that work together to form a structural complex or to carry out a particular biological function. An overview of the subsystems connected to this genome is given in Figure 1.

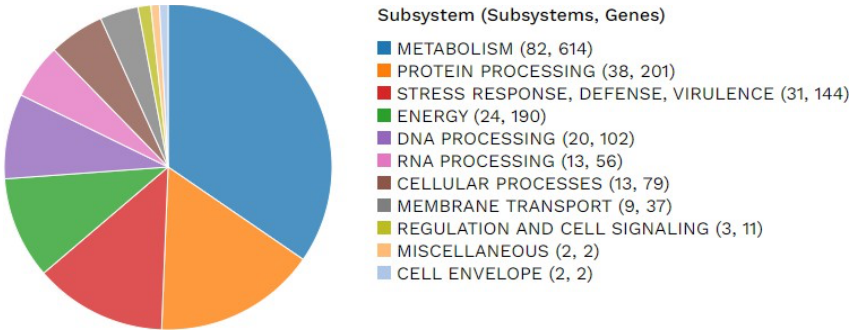

Figure 1 Subsystem category distribution statistic of *Staphylococcus arlettae* BK2L15. The Bacterial and Viral Bioinformatics Resource Center (BV-BRC) server has been used to display systemic feature and subsystem coverage. The pie chart depicts the subsystem distribution of *Staphylococcus arlettae* BK2L15, with metabolism (82 subsystems, 614 genes) dominating, highlighting its metabolic adaptability. Significant proportions of genes in protein processing and stress response, defense, and virulence suggest resilience and environmental adaptability, aligning with its genomic traits relevant to host interaction and survival.

Antimicrobial Resistance Genes (ARGs)

Comprehending the genetic foundation of resistance is essential for formulating effective strategies to counteract antimicrobial resistance (AMR). Table 3 presents the different mechanisms through which bacteria can develop resistance to antibiotics. These genes, which are also known as ARGs (antimicrobial resistance genes), confer resistance via various biological mechanisms.

Table 3 Antimicrobial Resistance Genes of *Staphylococcus arlettae* BK2L15

AMR Mechanism	Genes
Antibiotic inactivation enzyme	*fosB, mph(C) family*
Antibiotic resistance gene cluster, cassette, or operon	*tcaA, tcaB, tcaB2, tcaR*
Antibiotic target in susceptible species	*alr, ddl, fusA, tufA/tufB, folA, dfr, folP, gyrA, gyrB, inhA, fabI, ileS, kasA, MurA, rho, rpoB, rpoC, rpsJ, rpsL*
Antibiotic target protection protein	*msr(A)*
Efflux pump conferring antibiotic resistance	*ebrB, norA, ykkCD*
Gene conferring resistance via absence	*gidB*
Protein altering cell wall charge conferring antibiotic resistance	*gdpD, mprF, pgsA*
Regulator modulating expression of antibiotic resistance genes	*bceR, bceS, liaF, liaR, liaS*

Phylogenetic tree of taxonomy

This phylogenetic tree represents the evolutionary relationships among different species and subspecies of *Staphylococcus*. Each branch in the tree connects different Staphylococcus species or subspecies, indicating their genetic relatedness based on sequence data, likely from whole genome sequencing. *S. arlettae* BK2L15 isolate is closely related to *Staphylococcus arlettae* NCTC 12413. Both are part of the same clade, meaning they share a recent common ancestor and are genetically similar as clearly describes in Figure 2. The color-coded blocks associated with *S. arlettae* BK2L15 and *S. arlettae* NCTC 12413 indicate that they belong to the same species cluster and possibly the same subspecies cluster. This clade represents a closely related group within the *S. arlettae* species, suggesting that *S. arlettae* BK2L15 shares significant genetic similarity with the NCTC 12413 strain. The high bootstrap value and the similar associated genomic data (G+C content, genome size, protein count) reinforce the idea that these two isolates are very closely related.

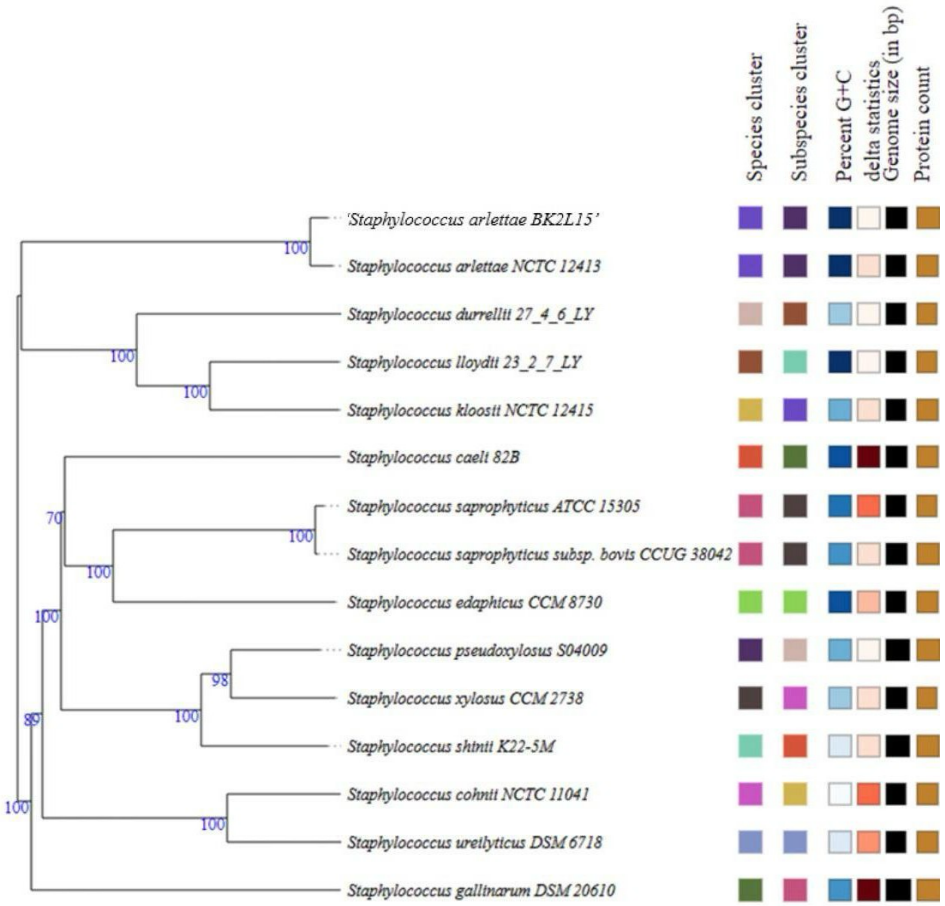

Figure 2 Phylogenetic tree of taxonomy of *Staphylococcus arlettae* BK2L15. The phylogenetic tree was constructed using Type (Strain) Genome Server (TYGS). Phylogenetic tree shows the evolutionary relationships of *Staphylococcus arlettae* BK2L15 with related *Staphylococcus* species, based on species clusters, GC content, genome size, and protein count. Closely grouped with *S. arlettae NCTC 12413*, BK2L15 highlights genetic similarities within the species. This study focuses on characterizing BK2L15, a livestock-associated isolate, to explore its genomic features, antimicrobial resistance mechanisms, and evolutionary adaptations. The tree underscores BK2L15's relevance within the genus, aiding in understanding its ecological role and contribution to antimicrobial resistance.

DISCUSSION

The role of *Staphylococcus arlettae* in diverse environments, including its potential association with bovine mastitis, remains underexplored. Studies on non-*aureus* staphylococci (NAS) have demonstrated strain-specific pathogenicity and the influence of environmental factors on microbial distribution, suggesting similar mechanisms may apply to *S. arlettae*. Despite these insights, comprehensive datasets linking *S. arlettae* to mastitis are lacking, and little is known about its survival strategies and interactions with host immune systems. Notably, this study marks the first report of *S. arlettae* isolated from a cow nasal swab in Malaysia, harboring the *mecA* gene determinant and exhibiting multidrug resistance (MDR).

Nasal carriage of Staphylococcus species may serve as a reservoir for pathogens causing mastitis in ruminants, with non-aureus staphylococci (NAS) like *Staphylococcus haemolyticus* and *Staphylococcus chromogenes* commonly found in bovine-associated habitats, including nasal passages, milk, and teat apices (Wuytack et al., 2020). These NAS isolates, particularly in quarters with elevated somatic cell counts, suggest a link between nasal colonization and intramammary infections. The adaptation of coagulase-negative staphylococci (CoNS) to sub-inhibitory disinfectant concentrations, leading to increased antimicrobial resistance and biofilm formation, further supports their persistence and potential transition to the mammary gland (Turchi et al., 2020; Marzoli et al., 2021). Additionally, airborne transmission of staphylococcal species via bioaerosols in livestock environments highlights another pathway for nasal carriers to contribute to mastitis (Riccardi et al., 2021). Despite these findings, more longitudinal studies are needed to establish a direct causal relationship between nasal colonization and mastitis development.

According to GenBank NCBI, only 59 whole genome sequences of *S. arlettae* have been deposited as of July 1, 2024. The first complete genome sequence, isolated from a biological laboratory environment at Hokkaido University, Japan, was published on June 3, 2019 (Yu et al., 2019). Notably, the first draft genome of *S. arlettae* linked to bovine mastitis, a significant concern in dairy cattle, has also been detected in human oral surgical sites, suggesting its involvement in surgical site infections (Han et al., 2022; Kherdekar et al., 2023). The development of bacteriophage-based biosensors further underscores the need to monitor this bacterium due to its pathogenic potential (Bhardwaj et al., 2016).

The *S. arlettae* strain BK2L15 was identified using MALDI-TOF, with a high-confidence score of 2.09. The detection of the *mecA* gene in this strain indicates methicillin resistance, as the gene encodes the alternative penicillin-binding protein PBP2a, conferring resistance to methicillin and other β-lactam antibiotics. This resistance poses significant clinical challenges, limiting treatment options. While Chanayat et al. (2021) classified *S. arlettae* strains harboring the *mecA* gene as SCCmec type V, our study was unable to determine the SCCmec type for BK2L15 due to incomplete genome sequencing data caused by low coverage of raw reads.

The structural diversity of SCCmec elements, as identified by Roy et al. (2024) in *S. aureus* and *S. argenteus*, underscores the need to investigate their variations in CoNS like *S. arlettae*. Studies show that SCCmec elements often integrate into mobile genetic platforms, enhancing their dissemination across bacterial populations (Lanza et al., 2015; Gómez-Sanz et al., 2021). While SCCmec typing has elucidated resistance mechanisms in *S. aureus*, its application in less-studied species remains underexplored. For instance, *S. arlettae* may possess unique SCCmec variants with novel resistance profiles, yet data is sparse, necessitating focused genomic surveillance (Gómez-Sanz et al., 2021; Abdullahi et al., 2023; Roy et al., 2024).

Antibiotic susceptibility testing of *S. arlettae* BK2L15, performed using the Kirby–Bauer disc diffusion method, revealed resistance to erythromycin, cefoxitin, fusidic acid, oxacillin, penicillin, azithromycin, and amoxicillin. However, the strain was susceptible to ciprofloxacin, tigecycline, tetracycline, and other antibiotics. These findings align with Andreis et al. (2017), who also reported penicillin

resistance in *S. arlettae* due to a novel β-lactamase operon, further complicating treatment options. A multiple antibiotic resistance (MAR) index of 0.4 for BK2L15 indicates a substantial resistance burden, highlighting the critical need for targeted antibiotic use and robust antimicrobial stewardship.

The genome assembly of BK2L15 yielded a total length of 2,699,512 base pairs with a GC content of 33.5% and two contigs. The assembly's N50 value of 2,668,290 base pairs suggests high-quality sequencing. Genome annotation identified 2,915 protein-coding sequences (CDSs) and 22 ribosomal RNA (rRNA) genes. Functional analysis of the genome highlighted a focus on metabolism, protein processing, and stress response mechanisms, reflecting the bacterium's adaptability and survival strategies. In contrast, categories such as regulation and signaling were less represented, suggesting reduced complexity in these processes.

The genome contains a diverse array of antibiotic resistance genes (ARGs) and regulatory elements contributing to multidrug resistance. Notable ARGs include *fosB* and *mph(C)*, which confer resistance to fosfomycin and macrolides (Argudín et al., 2015; Vázquez et al., 2023) as well as *tcaA-tcaB-tcaR* operons associated with teicoplanin resistance. Additionally, target modification genes such as *gyrA* and *fabI*, which mediate resistance to fluoroquinolones and triclosan (Couto et al., 2014). Efflux pumps like *norA* and cell wall modification genes such as *gdpD* and *mprF* further enhance resistance (Phillips-Jones and Harding, 2018; de Morais Oliveira-Tintino et al., 2022; Hillman, 2022). Regulatory genes such as *bceR* and *liaF* further enhance resistance by controlling gene expression, collectively equipping the strain to withstand intense antibiotic pressure (Weidemüller et al., 2021). Independent studies have also identified several multidrug efflux pumps (e.g., norA) coding genes as well as other genes related to resistance to antibiotics such as chloramphenicol (e.g., fexA), tetracycline (e.g., tetL), and erythromycin (e.g., msrA, mphC) in the genomes of SAR strains isolated from chicken farm and dairy herds affected by mastitis (Xu et al., 2015; Andreis et al., 2017; Liu et al., 2017; Nobrega et al., 2018)

Phylogenetic analysis revealed that *S. arlettae* BK2L15 shares a close evolutionary relationship with *S. arlettae* NCTC 12413, supported by a high bootstrap value of 100. Both strains cluster within the same species group, while *S. durrellii* and *S. lloydii* form distinct subspecies clusters. These findings confirm the genetic relatedness of BK2L15 to other *S. arlettae* strains and its position within the broader phylogenetic framework of staphylococcal species.

Staphylococcus arlettae has significant implications in veterinary and clinical settings, contributing to intramammary infections, antimicrobial resistance (e.g., *blaARL*, *mecA*), and zoonotic risks. It poses challenges to treatment, threatens farm productivity, and may facilitate horizontal resistance gene transfer to pathogens like *S. aureus*. Advanced diagnostics (e.g., biosensors) and its roles in arsenic bioremediation and plant growth promotion highlight its ecological and biotechnological relevance. Further research is needed to understand its pathogenicity, resistance mechanisms, and broader applications.

CONCLUSION

In conclusion, the findings from this study provide critical insights into the genetic and antimicrobial resistance profiles of *S. arlettae* BK2L15, the first reported isolate from a cow nasal swab in Malaysia carrying the *mecA* gene. The high-quality genome assembly and identification of multiple resistance genes underscore the strain's multidrug-resistant nature and its potential clinical impact. The phylogenetic analysis further situates BK2L15 within a closely related species cluster, highlighting its evolutionary significance. These results emphasize the need for ongoing surveillance and targeted interventions to manage and mitigate the

risks associated with this emerging pathogen in both veterinary and human health contexts.

ACKNOWLEDGEMENTS

We would like to express our sincere gratitude to Fundamental Research Grant Scheme (FRGS), Ministry of Higher Education of Malaysia under FRGS/1/2020/WAB04/UNISZA/03/2 for providing the financial support necessary to conduct this research. We also extend our appreciation to Universiti Sultan Zainal Abidin for providing the facilities and technical assistance essential for this study.

AUTHOR CONTRIBUTIONS

These authors contribute equally to the work

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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How to cite this article;

Puteri Nazurah Megat Zakaria, Suhaili Zarizal, Noor Muzamil Mohamad, Soo Sum Lean, Mohd Nasir Mohd Desa, Erkihun Aklilu and Chew Chieng Yeo. Draft genome sequences of multidrug resistant *Staphylococcus arlettae* isolated from cow nasal swab at Kelantan Malaysia Farm. Veterinary Integrative Sciences. 2026; 24(1): e2026004-1-12.